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Accuracy and sampling error of two age estimation techniques using rib histomorphometry on a modern sample

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ABSTRACT

Most age estimation methods are proven problematic when applied in highly fragmented skeletal remains. Rib histomorphometry is advantageous in such cases; yet it is vital to test and revise existing techniques particularly when used in legal settings (Crowder and Rosella, 2007). This study tested Stout & Paine (1992) and Stout et al. (1994) histological age estimation methods on a Modern Greek sample using different sampling sites.

Six left 4th ribs of known age and sex were selected from a modern skeletal collection. Each rib was cut into three equal segments. Two thin sections were acquired from each segment. A total of 36 thin sections were prepared and analysed. Four variables (cortical area, intact and fragmented osteon density and osteon population density) were calculated for each section and age was estimated according to Stout & Paine (1992) and Stout et al. (1994).

The results showed that both methods produced a systemic underestimation of the individuals (to a maximum of 43 years) although a general improvement in accuracy levels was observed when applying the Stout et al. (1994) formula. There is an increase of error rates with increasing age with the oldest individual showing extreme differences between real age and estimated age.

Comparison of the different sampling sites showed small differences between on the estimated ages suggesting that any fragment of the rib could be used without introducing significant error. Yet, a larger sample should be used to confirm these results.

Key words: Forensic Anthropology, age estimation, Rib histomorphometry, sampling error

INTRODUCTION

Age estimation of skeletal remains associated with forensic cases requires an appropriate choice of methodology use to meet specific legal expectations related to expert witness testimony and peer reviewed criteria [1]. Therefore, scientific methods used should be standardised in peer reviewed publications before they can be applied to court cases [2]. The vast majority of available age estimation methods specific to adult skeletal remains rely on degenerative changes to bone joint surfaces and are employed using macroscopic observation techniques [e.g. 3,4,5,6]. Most of these methods are not suitable for highly fragmented remains with incomplete bone inventory; remains that are often encountered by forensic anthropologists [7]. As a result age assessment using bone fragments examined under the microscope may provide age estimation answers. As with any age assessment the accuracy of the result depends in part to training and experience.

Microscopic techniques focus on the variation of micro-anatomical patterns in the bone cortex such as cortical area and a count of bone features (for example, secondary osteons) [8,9]. Although the reliability and feasibility of histological methods use to estimate age have been demonstrated [10]; the disadvantages –e.g. the destructive and time-consuming nature of the technique, the necessity for specialised equipment and training and the required observer's experience are considered significant drawbacks for its applications in forensic investigations [11]. The alternative use of manual thin sectioning techniques (12,13) instead of expensive equipment can however reduce the cost significantly. Thus, histological analysis still has potential as the skeleton is subjected to inter-population variation in metabolism and bone microstructure is a record of past metabolic events.

The development of methodologies based on bone histomorphometry has increased in numbers and bone regions during the last 45 years. Some of these studies have focused on the differentiation between animal versus human remains [14,15]; the analysis of pathological conditions and their impact on bone microstructure [16,17]; and histological determination of age at death [7,18,19,20,21]. The age assessment rests on the observation of changes in cortical bone microstructure features during the life of the individual. The methodology is based on the remodelling process in which older bone is replaced through the activity of bone cells: osteoblasts or bone forming cells, and osteoclasts or bone resorbing cells [22]. They both work in coordination resulting in the basic structural units known as secondary osteons or Haversian systems [23]. As remodelling occurs throughout life, the changes experienced by these units will constitute the basis of aging methods and the principle applied in age estimation techniques.

The first aging technique using histological features was developed by Kerley in 1965. He used bone cross sections from the femur, tibia and fibula. He created multiple age regression formulas per bone using quantitative variables correlated to age [9]. History has shown that only the Kerley equation using intact osteon numbers of femoral bone remains in common practice by anthropologists [24]. Since 1965, the femur has been subjected to attempts to improve the predictive power of histological assessment for age estimation [25,26]. As part of this process several attempts also developed equations that were population specific to improve the equations accuracy [27,28,29].

Since 1992, a number of histomorphometric studies have been carried out on ribs using micro-anatomical variables in the attempt to determine higher

accuracy rate in age prediction [7, 30]. In doing so new variables based on osteon features were introduced in creating age estimation equations [7,20,31,32, 33].

As a result of the early work done on ribs [7], they are now often used as they can be easily obtained during standard autopsy procedures without further dissection as it would be required for sampling long bones. In addition, the available data on normal and pathological rib physiology [7,17,34]; and the fact that the rib allows for a full examination of the entire section under the microscope, make them an ideal bone to work with on future research endeavours. The fragmented nature of archaeological ribs, however, can lead to poor identification to their location and/or rib number which potentially can cause problems of sampling rib micro-features [e.g. 1]. This matter is one of the research questions we will address in this study. A second goal is to test the applicability of two existing methods using rib criteria to assess age using a modern autopsy sample. The last objective of this study concerns the estimation of inter-observer error rates in quantifying micro-anatomical features (fragmentary and intact osteons) on rib thin sections.

One of the first studies using rib histomorphometry for age estimation of unknown human remains was carried in the early 1980's [7]. The sample consisted of 40 individuals; the middle third of the 6th left rib was processed following the standards of histological preparation outline by Stout and Paine [7]. To test their rib equation the authors applied it to 12 rib test samples from autopsies. Their results showed that the standard errors from actual age to estimated age were -2.7 to +9 years for the rib, and -8.1 to +20.6 for the clavicle. The combined formula gave an error of -2.5 to + 14.5 years. Most of the individuals fell within 95% of confidence interval for the estimated ages [7].The lower mean absolute differences between

actual and predicted age was for the combined formula suggesting that histological age estimation yields more accurate results when both skeletal elements are examined. It is worth mentioning the new variable created by the authors: osteon population density (OPD) as a combination of intact and fragmentary osteons; both are the product of cortical remodelling. OPD has become a standard variable used by histological working on age assessment in an anthropological context [24]. This new parameter has been extensively used in recent histological research due to its high correlation with age [20].

There have been several attempts to use both macro and micro-anatomical rib features to estimate age-at-death. Stout et al. [35] used sections extracted from the sternal end of the 4th rib to estimate age at death incorporating the combination of two age related changes: morphological changes and microscopic bone structures. The first method was based on İşcan et al.'s [3,36] technique for aging individuals through the sternal rib end and the microscopic approach was carried out by applying Stout and Paine's method [7]. Two estimation formulae were generated: one used histological features only and the second equation applied both micro and macro-assessment techniques. Multiple variables included in the equation provided more accurate results (SEE = 7.18) than each method applied separately. This suggests that the multifactorial approach is preferable when possible. Dudar et al. [30] also tested both techniques finding no statistically significant differences between them although more accurate results were produced by using the combined micro and macro variables.

The purpose of this paper is to report the results of the histological age estimates using both Stout and Paine [7] and Stout et al. [35] methods on a contemporary Greek sample. The objectives are the following:

- a) To test the accuracy of applying the two equations developed from US populations on a contemporary Greek sample and to verify if population specific formulae are required.
- b) To explore the effect of sampling error on age estimation on six different sampling sites along the rib in order to compare the accuracy levels between different sampling areas.

MATERIAL AND METHODS

Sample

The histological sample consists of six 4th left ribs (with known sex and age) from a Modern Cretan Collection [37]. Due to difficulties in finding complete ribs, the selection of the sample was limited in number of specimens and individual's profile; all the specimens selected were females and only the fourth rib was available for analysis. Although Stout and Paine's [7] technique was developed from the 6th rib, studies have shown that the methodology can be reliably applied on other ribs [1]. According to this publication, the histological findings of the fourth rib can be interchangeable with the sixth rib. With regards to known age-at-death, the sample represents an age range from 19 to 58 years old with a mean age of 36 years (*Table 1*).

Table 1. Rib sample and real age.

Rib N°	Age
Rib_53	19 y.o.
Rib_88	27 y.o.
Rib_28	29 y.o.
Rib_180	35 y.o.
Rib_194	46 y.o.
Rib_6	58 y.o.
Mean	36 y.o.

Preparation of thin sections and data acquisition

The preparation of the thin sections is described in Stout and Paine [7] and Paine [38]. The sternal and vertebral ends of each rib were removed using a manual semiautomatic saw and three areas of each rib (superior, inferior and lateral) were color-coded with the purpose of identifying the location area of the bone once the rib sections were processed. Each rib was divided into three equal segments identified as proximal, middle and distal. The 18 fragments were embedded in Epo Thin resin (Buehler™) to ensure the preservation of both cortical and trabecular bone during the cutting process. Once the embedding material was cured, a Buehler™ IsoMet 1000 Precision Saw (sectioning machine) was used for cutting approximately 1 mm sections from the resin blocks. Two sections from each proximal, middle and distal segment were cut in order to have different sampling locations representing all the areas along the length of each rib (N=36).

The thin sections were glued onto frosted slides and prepared for histological analysis by grinding them to a thickness of 50-70 microns using a Buehler™ variable-speed grinding unit using 1200 grit paper. Once the histological features were observable, the bone wafers were mounted onto reading slides.

Histomorphometric data were collected using a binocular transmitted light standard research microscope (Olympus CH-2) at x100 and x200 magnification (x10 and x20 eyepieces and X 10 objective). Four histomorphometric variables as indicated by Stout and Paine [7] were assessed:

- Cortical Area: all areas of cortical bone contained within the microscopic fields read per section (in mm²).
- Intact Osteon: number of secondary osteons that have at least 90% of their canal intact.
- Fragmentary Osteon: number of secondary osteons that have less than 90% of their canal is present.
- Osteon Population Density (OPD): the sum of intact and fragmentary osteons divide by cortical area

Instead of using the Merz counting reticule to calculate cortical area as Stout and Paine [7] suggested, we up-dated the means for measuring cortical area by using digital handheld microscope (Dino Lite®) to capture images from the entire rib section (Figure 1). An open source software (ImageJ 1.48) was employed to measure the cortical bone area that was outlined manually (cortical area=complete rib section – medullary area) on the images. To do this we placed the thin section over a scale and calibrated the software with the Dino Lite microscope. OPD was calculated and was then used for estimating age-at-death according to Stout and Paine (F1) [7] and Stout et al. (F2) [35] (Table 2).

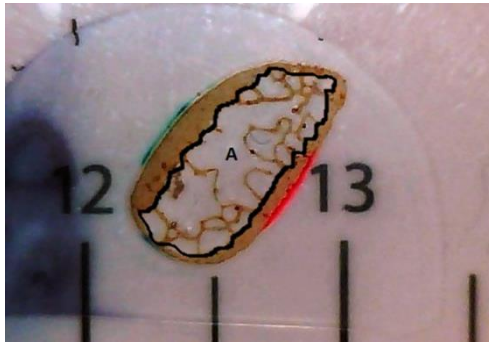


Figure 1: Image taken using Dino Lite® for measuring the cortical area; A (black outline) indicates trabecular area.

Table 2. Age predicting equations applied on the Greek sample.

Author / year	Methodology	Formulae	SEE
Stout and Paine (1992)	<i>Sixth rib using midshaft sampling area</i>	$\text{Ln} = 2.343 + 0.050877X$	3.9
Stout et al. (1994)	<i>Fourth rib using proximal sampling area</i>	$\text{Age} = 18.389 - 0.731 * (\text{OPD}) + 0.110 * (\text{OPD})^2$	10.43

Inter-observer error was determined following TEM analysis (technical error of measurement) in order to assess the level of agreement in osteon counting between observers (RRP and JGGD) for 18 sections. TEM was calculated by taking the square root of the sum of the squares of the assessment differences and dividing the result by twice the total number of observation made [39]. The relative TEM was also calculated; this value expresses from 0 to 1 the proportion of variance unrelated to measurement error [40]. The two observers had a different level of experience on bone histomorphometry. JGGD had three years of experience in bone histological analysis as researcher while RRP had over 35 years of experience as forensic histologist and he has developed one of the two techniques tested here [7] back in 1992.

Results

Inter-observer error

In order to test the reliability and feasibility of the methodology, two observers assessed part of the sample (N=18). The mean difference between observations (TEM) and the relative TEM are illustrated in Table 3. Overall, the absolute mean difference between observers' counts is very low. The difference between them is 6 intact osteons, 5 fragmentary with a difference of 4 total osteons. The calculation of the relative TEM gave promising results; the lowest relative TEM value (83%) was reported for fragmentary osteons and the total osteon counting encountered the best value with 99% of the variance free from measurement error indicating agreement between observers.

Table 3. Estimation of interobserver error with the calculation of Relative TEM for Intact, Fragmented, Total Number of Osteons and OPD.

Specimen	Intact Osteons			Fragmented osteons			Total number of osteons			OPD		
	OB1	OB2	Relative TEM	OB1	OB2	Relative TEM	OB1	OB2	Relative TEM	OB1	OB2	Relative TEM
R53D1	105	103	1.36	15	14	4.88	120	117	1.79	5.77	5.62	2.07
R53M1	88	92	1.32	22	14	31.43	100	106	4.12	4.07	4.32	8.18
R53M2	126	124	1.13	34	34	0.00	160	158	0.89	7.03	6.94	0.89
R53P1	240	238	0.59	29	26	7.71	269	264	1.33	7.72	7.58	1.33
R53P2	168	174	2.48	41	42	1.70	209	216	2.33	7.73	7.99	2.33
R88D1	46	44	3.14	5	3	35.36	51	47	5.77	2.59	2.39	5.77
R88M1	119	111	4.92	17	16	4.29	136	127	4.84	4.67	4.36	4.84
R88M2	144	159	7.00	30	16	43.04	174	175	0.41	6.39	6.42	0.41
R88P1	156	166	4.39	32	28	9.43	188	194	2.22	6.14	6.34	2.58
R88P2	172	193	8.14	50	37	21.13	222	230	2.50	6.98	7.23	2.50
R28D 1	101	102	0.70	24	23	3.01	125	125	0.00	5.87	5.87	0.00
R28D2	110	118	4.96	25	19	19.28	135	137	1.04	5.57	5.66	1.04
R28M1	165	182	6.93	43	25	37.44	208	207	0.34	6.40	6.37	0.34
R28P1	138	130	4.22	30	34	8.84	168	164	1.70	4.85	4.73	1.70
R28P2	186	189	1.13	49	43	9.22	235	232	0.91	6.95	6.87	0.91
R180P1	119	123	2.34	49	53	5.55	168	176	3.29	6.54	6.85	3.29
R194M1	140	139	0.51	19	15	16.64	159	154	2.26	7.18	6.95	2.26
R6P1	112	110	1.27	16	21	19.11	128	131	1.64	8.37	8.56	1.64

Comparison of the two histological methods

Error rates produced by applying the two formulae in the sections from the middle fragments of the ribs are summarised in Table 4. In all cases age was underestimated up to -38 years for F1 and -43 years for F2 with the exception of rib 53 (19 years old) which yielded an error range from -2 to +1 using F2. In general, F2 [35] gave relatively lower error range compared to F1 [7]. Both formulae exhibited higher error rates with increasing age. Overall, the error rates produced by both histological methods did not fall within the error rates reported by the original techniques with the exception of the 19 years old individual F1 [7] and two youngest individuals for F2 [35].

Table 4. Error rates using Stout et al. (1994) and Stout and Paine (1992) equations for age estimation.

Specimen	Age	F2 error range	F1 error range
53	19	-2 to +1	-17 to -3
88	27	-10 to -9	-16 to -12
28	29	-11 to -12	-16 to -15
180	35	-18 to -15	-22 to -19
194	46	-31 to -28	-28 to -23
6	58	-43 to -37	-38 to -31

Intra-costal age estimates comparing the two histological formulae

When different samples sites were used for age estimation, F1 [7] displayed a mean error ranging from a minimum of 21% for the younger individual to a maximum of 66% for the oldest individual (Table 5). The two youngest individuals obtained the best estimates from the proximal sections; for the 35 years old individual both middle and proximal sections were the most accurate; and the best results for the oldest individual were produced by the middle thin sections.

As seen in table 5 the difference between two age estimates from sections of the same rib and the deviation from the mean age estimate are in most cases much smaller than the difference between the age estimate and the real age. For example for the individual of 27 years old (rib 88) the age estimate ranged between 11.87 and 15.11 y.o. (Mean=13.94+/-1.21 y.o., Table 6). This means that the absolute error (-11.89) of the best age estimate (Distal=15.11 y.o.) is larger than the error introduced by using a different sampling site. The largest difference between two age estimates is 3.24 years and the standard deviation from the mean is 1.21 years. The same observational pattern is exhibited by all rib samples except for rib 53; where the absolute error of the best age estimate (-2.55 years) is smaller compared to the largest difference between two age estimates (3.64 years) (Table 6).

Table 5. Summary table of Stout and Paine (1992) and Stout et al. (1994) error rates. * %= 100*Mean E/Real Age.

Rib Nº	Age	<i>Stout and Paine (1992)</i>		<i>Stout et al.(1994)</i>	
		Mean Error	Mean error %	Mean Error	Mean error %
Rib_53	19	-4.17	-21.95	-0.15	-0.80
Rib_88	27	-13.05	-48.33	-8.92	-33.05
Rib_28	29	-14.87	-51.28	-11.00	-37.92
Rib_180	35	-19.99	-57.10	-16.09	-45.97
Rib_194	46	-29.45	-64.01	-24.97	-54.29
Rib_6	58	-38.64	-66.63	-31.96	-55.11

F2 [35] also presented a systemic underestimation of the individuals with only one of the distal sections from the youngest individual slightly overestimating real age (over 1 year from real age). The mean error rate varied from 1% for the 19 years old specimen to a maximum of 55% for the oldest individual (Table 5). Similarly to F1 the results provided by each rib segment showed no particular trend: age estimates of the youngest individual were more accurate for the proximal

segments, although there was high variation between rib segments for the rest of the individuals. An increasing error rate was observed with increasing age although the age difference is not as high as it was observed for the other histological method (58 years old individual being estimated as 42 years old). The difference between two age estimates from sections of the same rib and the deviation from the mean age estimate are in most cases much smaller than the difference between the age estimate and the real age as it was observed for F1 [7]. For example for the individual of 27 years old (rib 88) the age estimate ranged between 17.23 and 18.65 y.o. (Mean=18.08+/-0.68 y.o.) (Table 6). This means that the absolute error (-8.35) of the best age estimate (Proximal=18.65 y.o.) is larger than the error introduced by using different sampling site. The largest difference between two age estimates is 1.42 years and the standard deviation from the mean is 0.68 years.

Table 6. Age estimates using Stout and Paine (1992) and Stout et al. (1994) histological formulae.

		Age estimation Stout and Paine (1992)							
Rib Nº	Age	D (1)	D (2)	M(1)	M(2)	P(1)	P (2)	Mean	SD
Rib_53	19	13,96	16,45	12,81	14,88	15,42	15,43	14,83	1,28
Rib_88	27	11,87	15,11	13,2	14,4	14,23	14,85	13,94	1,21
Rib_28	29	14,03	13,82	14,41	14,33	13,32	14,83	14,12	0,52
Rib_180	35	14,43	13,97	16,24	15,32	14,52	15,57	15,01	0,85
Rib_194	46	18,23	17,62	15,02	17,37	15,68	15,39	16,55	1,35
Rib_6	58	20,32	19,03	21,89	19,44	19,48	15,93	19,35	1,96
		Age estimation Stout et al. (1994)							
Rib Nº	Age	D (1)	D (2)	M(1)	M(2)	P(1)	P (2)	Mean	SD
Rib_53	19	17,83	20,72	17,24	18,68	19,3	19,31	18,85	1,23
Rib_88	27	17,23	18,94	17,38	18,21	18,05	18,65	18,08	0,68
Rib_28	29	17,89	17,73	18,21	18,14	17,43	18,63	18,01	0,42
Rib_180	35	18,23	17,84	20,41	19,18	18,31	19,49	18,91	0,96
Rib_194	46	23,68	22,6	18,81	22,17	19,64	19,27	21,03	2,04
Rib_6	58	27,8	25,19	31,19	25,99	26,08	19,98	26,04	3,66

*D=distal segment; M=midshaft segment; P=proximal segment. First section (1) and second section (2) from the same segment

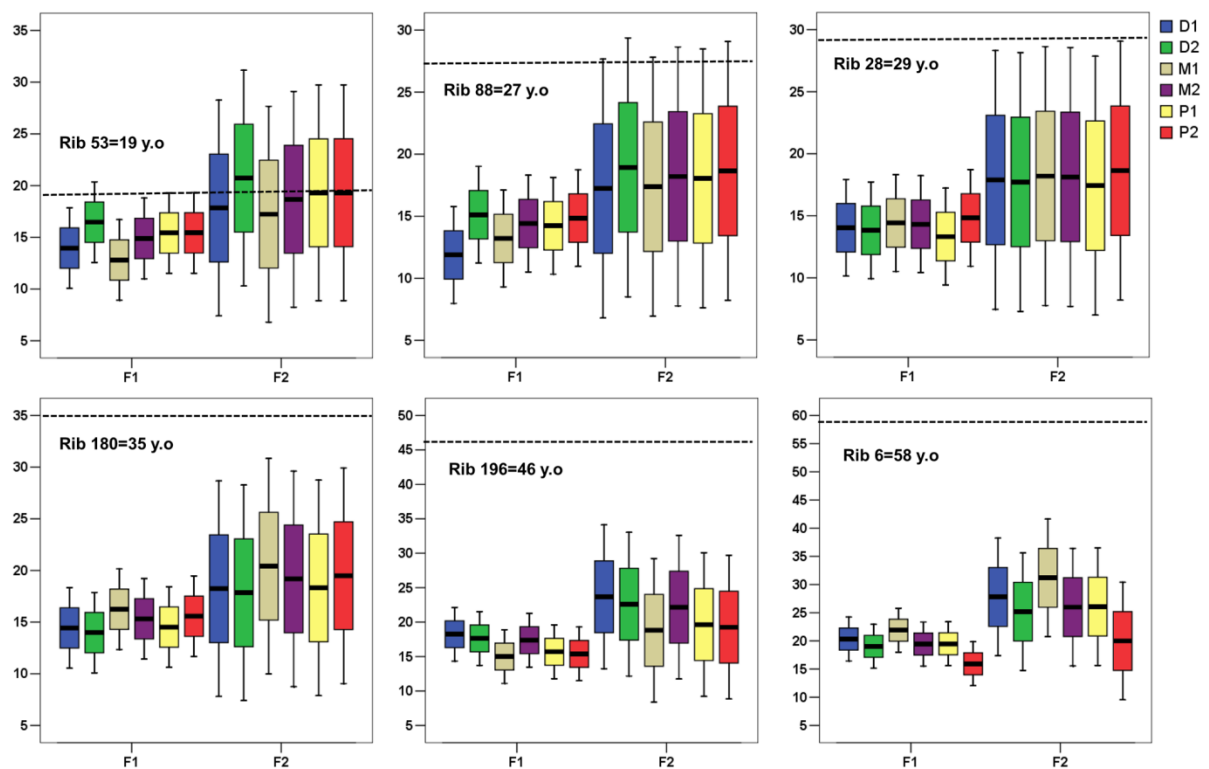


Figure 2: Illustration of age ranges for F1 and F2 (including originally reported error rates) in relation to the real age (horizontal line) for each segment/section. D=distal, M=middle, P=Proximal fragment.

DISCUSSION

The reliability and feasibility of the histological methodologies used within the forensic context in order to meet the legal standards of expert witness testimony in court room requires considerable review [1]. The choice of age assessment methods depends on the specific circumstances of a case. Well trained skeletal histologists should be consulted when micro-anatomical features are used to estimate age-at-death. According to Ritz-Timme et al. [41] bone histology applied for age estimation of modern human remains reported error rates of ± 5 to -12 years for most methods. Aiello and Molleson [42] agree that both macroscopic and microscopic aging methodologies should be representative of “age structure and/or

conditions” of the population from which the methodology was developed. Therefore, peer-reviewed population specific standards are desirable to assure accuracy of age estimations.

Test of the histological methods for the Greek population

The first objective of this project was to test if two aging methodologies generated from US reference samples produce accurate results when applied to a Greek sample. The results indicate that there is a systematic underestimation of all individuals for all six sections along the rib. The difference from real and estimated age ranges from 3 years to a maximum of 43 years for F1 [7] and from 1 year to a maximum of 39 years for F2 [35]. Age estimates produced by the two equations do not fall within the error rates reported by the original studies, with the exception of the estimates for the youngest individual using F1 [7] and the two youngest individuals for F2 [35].

It is observed that the average error rate in age estimation increases dramatically with age with the best results provided by the youngest age group. Once intact and fragmentary osteons reach asymptote they do not increase in number with age; herein, the evidence of bone remodelling is not visible anymore. For ribs, it can occur between 50-60 years old depending on the remodelling rate of the sample collection studied, and metabolic factors that affect the osteon size or cortical diameter [24]. The extremely high error rates produced by the 58 years old individual may be attributed to this phenomenon. Paine and Brenton [17] also observed that the rib equation under-aged a nutritionally challenged Black South African sample. They attributed this trend to poor diet affecting bone remodelling by decreasing the production of secondary osteons resulting in younger looking

than expected cortical bone. In this study both methods performed better for younger individuals than what was reported in by other studies [32]; yet, this needs to be confirmed by testing a larger sample.

Our preliminary results suggest that population specific standards are required for applying age histological age estimation standards in Greeks and there is a scope for expanding the sample to verify these results and create new reliable population specific equations. Several studies have already shown population variability in histological analysis confirming that population specific formulae produce more accurate results than using those formulae generated from a different reference sample [20, 43]. For instances, African Americans showed lower trabecular bone turnover than American whites from Unites States; producing high levels of error in age estimation [20, 44]. On the contrary, nineteen century Eskimos femoral micro-anatomical features exhibited greater turnover rates than a U.S. white sample obtaining overestimated age predictions [45].

Other studies have focused on applying existing femoral formulae on different populations. Ubelaker [46, 47] tested Kerley's methodology [9] on a Dominican mixed ethnic sample and found an error average up to 11 years. Fangwu [48] used the same formula on Modern Chinese femora with one third of the sample having errors greater than ± 10 years, confirming the necessity of a specific predicting equation for that population. A recent study carried out on clavicles from a Korean population found that their equations were no better that Stout and Paine [7] clavicle equations but they too suggest that validity of population specific techniques for accurately estimating age-at- death is preferred [49]. We intend to explore this by developing a new age predicting formula from a Greek rib sample to

verify whether the error rates produced in the current analysis is due to inter-population variability or not.

In addition to inter-population variability the use of specific ribs might have also affected the estimation of age in this study, specifically when applying Stout and Paine's formula [7] which was created using the 6th rib. Crowder and Rosella [1] carried out a research on whether other ribs apart from the sixth one can be used for estimating age at death without producing high error levels. The authors examined midshaft cross sections from the 3rd to the 8th rib from 20 cadavers. They found that the higher error was produced by the 8th rib –probably due to biomechanical remodelling caused by the absence of sternal attachment. The results showed that variation among ribs within the same individual produced certain bias, but all ribs produced consistent OPD values for age assessment; thus, other ribs apart from the 6th could be used for age estimation purposes and no error would be expected. Based on this assumption it could be argued that the source of error for our age estimates was not rib number although further studies are required to verify this statement.

There are other intrinsic factors affecting the accuracy of our results that need to be considered. Due to limitations in the sample, only female individuals were used for this study and this fact could have caused bias in the results because the original methods were based on pooled sexes. Some authors suggest that sex differences are crucial in the generation of age predicting equations [8,33] whilst others did not report any variation [7,9,26]. Women have an increase in bone remodelling rate after menopause [50] which would produce an overestimation of the actual age at death. Additionally, Burr et al. [51] noticed than secondary osteons

exhibited by older females were larger in size than those of males. This finding could have a direct effect on the accuracy of age estimation because in females a specific area of the bone cortex would accommodate fewer osteons. However, other studies have found no difference in osteon size between sexes [52]. While Paine and Brenton [17] found osteon size may reflect chronic metabolic conditions such as dietary deficiencies. These discrepancies in the results could be due to differences in environmental factors, physiological conditions specific to sex or sampling errors. If sex differences in histological analysis are assumed for the present study, the overall accuracy of age estimates would have been decreased by sex-related variation explaining the low performance of the formulae applied. The inclusion of male individuals in future research will elucidate if sexual dimorphism in histological microstructures does exist in the Greek population.

Although there was not specific clinical data available for the sample used in this project, the examination of pathological conditions affecting histological microstructures would have added a new perspective to our results. Stout and Paine's [7] predicting formula was tested by Paine and Brenton [17] on a sample of individuals suffering from pellagra. Age estimations were considerably lower than those obtained from the original autopsy sample. The results showed the impact of metabolic disturbances on the accuracy of histological methods and suggested that the consideration of these factors must be addressed when dealing with samples suspected of suffering from metabolic disorders. Furthermore, metabolic disturbances like osteoporosis which is frequent among women of advanced age [53,54]. Consequently, the alteration of bone remodelling patterns at all age ranges between sexes must be considered, and thus the histomorphometric variables used in the creation of aging equations need to be adjusted [34]. Although

they suggest this as a possible contributing factor no histological study so far has produced data that supports the need for a sex based age-at -death estimation equation.

Intra-costal variation and age estimation accuracy

The second objective of this work was to explore whether the effect of sampling sites on the accuracy of age estimates. Ribs are frequently fragmented and quite often it is not possible to identify the sampling area or even rib number. With regards to sampling area, previous research has demonstrated that site specific variability exists and that this should influence how we approach data collection schemes [7,25]. This hypothesis was tested by analysing two thin sections from the distal, medial and proximal regions of the fourth rib against two existing methods. Figure 2 compares the age estimates obtained for the six sampling sites of each rib to real age; the underestimation of all individuals is obvious and different accuracy levels obtained by the two histological formulae equation can be clearly observed.

Stout and Paine [7] original method was based on the middle third of the 6th rib but using this sampling location did not produce better results than the other sampling sites along the ribs. Stout et al. study [35] was developed using sections from the sternal end; yet, the equivalent sternal sampling area did not perform as it was expected. Moreover, comparison of the different sampling sites did not follow a specific pattern. Comparing age estimates variation between rib segments to the error produced by the formulae and using the sampling area used by the original methodology, it is clear that sampling location has little impact on the accuracy of the age estimation methods. The variation between estimates from the three different segments of the same rib only ranged for up to 4 years in both cases. For

the 58 year old individual F2 [35] produced an error as high as 12 years. The intra-segment variation using F2 [35] was found to be greater compared to F1 [7]. These results indicate that any segment of the rib could be used for age estimation due to the small error that it will produce in age assessment.

Up to date, there are no other histological studies, to our knowledge, testing the effect of sampling site along the rib on the accuracy of age estimation methods. One study reported variation within the same rib section [32]. The authors examined the external and internal cortex of the costochondral area of the fourth rib for age estimation. OPD counted in the internal cortex was the better variable when classifying the sample divided into three age-range groups. This assessment might be included in future research to test the potential of different sampling areas within the same thin cross section when assessing age at death.

With respect to long bones, serial sections have actually exhibited inconsistency in age estimation when they are other than the midshaft segment [55]. Other studies have reported femoral variation between several sub-areas within the anterior area [56] or regional variation on five different sampling areas along the midshaft [57]. These findings could be attributed to differences in bone loading and bone remodelling among long bones. Clearly mechanical loading experienced by the skeleton can accelerate the rate of intra-cortical modelling and remodelling producing histomorphometric differences in bone density both between bones and between individuals [51,58]. Although ribs are under a constant respiratory mechanical activity, they are of great value to avoid the variability caused by physical activity levels [33]. The fact that ribs are subjected to small weight bearing, and therefore less involved in biomechanical responses could explain the low

variation found between ribs segments in this sample. A larger sample should be used to further test sampling error variability and to verify these preliminary results.

Inter-observer error

Although not a primary goal in this study, the reliability and repeatability of the histological methods employed was tested by comparing the counting of two different observers. TEM and relative TEM were calculated for intact, fragmented and total osteons. Although the error for counting fragmented osteon counts between observers was quite high (17%), the error for total osteon count was only 1%. Since the formulae use the total number of secondary osteons for the age estimation it was concluded that there is agreement between the two assessments and subjectivity does not have an impact on the reliability and performance of the method. Our finding specific to inter-observer difference in identifying bone micro-anatomical features is in accordance with other studies [59]. And it points to one additional benefit when using the Stout and Paine equations [7], in the end, disagreements over intact and fragment osteons does not lead to inaccuracies in OPD values.

In summary our results indicate that Stout and Paine (7) and Stout et al (35) produced a systematic underestimation of age for the Greek sample suggesting that there is a need for population specific standards for more accurate age estimates. Due to the small sample size these results would need to be confirmed. The sampling site seems to have minimum effect on the performance of the histological age estimation methods tested here [7,35] as the error introduced by the use of a different sampling location is significantly lower compared to the error produced by

each method. Inter-observer error is very low suggesting that these techniques can be applied by individuals with reasonable level of training and research experience.

Conclusion

Due to evidentiary rules surrounding admissibility of forensic evidence, anthropological techniques applied to forensic case must be scrutinised to ensure accurate results (60). This includes publications that review methods and provide error rates that can be used in court as anthropologists are asked to discuss their findings. In this study, the Stout et al. [35] formula performed better in comparison to the Stout and Paine [7] formula on the Greek sample which can be attributed to intrinsic factors and small sample size. Our results suggest that reference bones play an important role in terms of reliability and accuracy, and that population specific standards might be required to avoid an increase in error rates. This study also provides new information about the possibility of using alternate sampling areas in ribs for estimating age at death. In view of this promising outcome, it seems necessary to expand the sample size to verify these preliminary results.

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References

1. Crowder C, Rosella L. Assessment of intra- and intercostal variation in rib histomorphometry: its impact on evidentiary examination. *J Forensic Sci* 2007; 52: 271–6.
2. Christensen AM, Crowder CM. Evidentiary Standards for Forensic Anthropology. *J Forensic Sci* 2009; 54: 1211–6.
3. İşcan MY, Wright RK. Age estimation from the rib by phase analysis: White males. *J Forensic Sci* 1984a; 29(4):1094-104.
4. Lovejoy CO, Meindl RS, Pryzbeck TR, Mensforth RP. Chronological metamorphosis of the auricular surface of the ilium: A new method for the determination of adult skeletal age at death. *Am J Phys Anthropol* 1985; 68: 15-28.
5. Meindl RS, Lovejoy CO. Endocranial suture closure: A Revised method for the determination of skeletal age at death based on the lateral-anterior sutures. *Am J Phys Anthropol* 1985; 68: 57-66.
6. Brooks S, Suchey JM. Skeletal age determination based on the os pubis: A comparison of the Acsádi-Nemeskéri and Suchey-Brooks methods. *J Hum Evol* 1990; 5: 227-38.
7. Stout SD, Paine RR. Brief communication: histological age estimation using rib and clavicle. *Am J Phys Anthropol* 1992; 87: 111–5. doi:10.1002/ajpa.1330870110
8. Ericksen MF. Histologic estimation of age at death using the anterior cortex of the femur. *Am J Phys Anthropol* 1991; 84: 171–9.
9. Kerley ER. The microscopic determination of age in human bone. *Am J Phys Anthropol* 1965; 23: 149–63.
10. Martrille L, Irinopoulou T, Bruneval P, Baccino E, Fornes P. Age at Death Estimation in Adults by Computer-Assisted Histomorphometry of Decalcified Femur Cortex. *J Forensic Sci* 2009; 54: 1231–7.
11. Klepinger LL. *Fundamentals of Forensic Anthropology*. New Jersey: John Wiley & Sons; 2006.
12. Maat GJR, Van Den Bos RPM, Aarents MJ. Manual Preparation of Ground Sections for the Microscopy of Natural Bone Tissue: Update and Modification of Frost's 'Rapid Manual Method'. *Int J Osteoarchaeol* 2001; 11:366-374.
13. Beauchesne P, Saunders S. A Test of the Revised Frost's 'Rapid Manual Method' for the Preparation of Bone Thin Sections. *Int J Osteoarchaeol* 2006; 16: 82–87.

14. Crescimanno A, Stout SD. Differentiating fragmented human and nonhuman long bone using osteon circularity. *J Forensic Sci* 2012; 57: 287–94.
15. Hillier ML, Bell LS. Differentiating Human Bone from Animal Bone: A Review of Histological Methods. *J Forensic Sci* 2007; 52: 249–63. doi: 10.1111/j.1556-4029.2006.00368.x
16. Cook M, Molto E, Anderson C. Possible case of hyperparathyroidism in a Roman period skeleton from the Dakhleh Oasis, Egypt, diagnosed using bone histomorphometry. *Am J Phys Anthropol.* 1988; 75: 23–30.
17. Paine RR, Brenton BP. Dietary Health Does Affect Histological Age Assessment: An Evaluation of the Stout and Paine (1992) Age Estimation Equation Using Secondary Osteons from the Rib. *J Forensic Sci* 2006; 51: 489-92.
18. Kerley ER, Ubelaker DH. Revisions in the microscopic method of estimating age at death in human cortical bone. *Am J Phys Anthropol* 1978; 49: 545–6.
19. Thompson DD, Galvin CA. Estimation of age at death by tibial osteon remodeling in an autopsy series. *Forensic Sci Int* 1983; 22: 203-11.
20. Cho H, Stout SD, Madsen RW, Streeter MA. Population-specific histological age-estimating method: a model for known African-American and European-American skeletal remains. *J Forensic Sci* 2002; 47: 12–8.
21. Streeter M., Stout S.D., Trinkaus E., Stringer C.B., Roberts M.B., Parfitt S.A. Histomorphometric age assessment of the boxgrove 1 tibial diaphysis. *J. Hum. Evol.* 2001; 40(4): 331–338
22. Stout SD, Crowder C. Bone Remodelling, histomorphology and histomorphometry. In: Crowder C, Stout S, editors. *Bone Histology: An anthropological perspective*, USA: CRC Press, Boca Raton; 2012, p. 1-22.
23. Gosman JH. Growth and Development: morphology, mechanisms and abnormalities. In: Crowder C, Stout S, editors. *Bone Histology: An anthropological perspective*, USA: CRC Press, Boca Raton; 2012, p. 23–44.
24. Robling GA, Stout SD. Estimation of Age at Death Using Cortical Bone Histomorphometry. In: Katzenberg MA, Saunders SR, editors. *Biological Anthropology of the Human Skeleton*. New Jersey: John Wiley & Sons; 2008, p. 149–182.
25. Ahlqvist J, Damsten OA. Modification of Kerley's method for the microscopic determination of age in human bone. *J Forensic Sci* 1969; 14: 205–12.
26. Singh IJ, Gunberg DL. Estimation of age at death in human males from quantitative histology of bone fragments. *Am J Phys Anthropol* 1970; 33: 373–81.

27. Maat GJR., Maes A, Aarents MJ, Nagelkerke NJD. Histological age prediction from the femur in a contemporary Dutch sample. The decrease of nonremodeled bone in the anterior cortex. *J Forensic Sci* 2006; 51: 230–7.
28. Stout SD, Gehlert SJ. The relative accuracy and reliability of histological aging methods. *Forensic Sci Int* 1980; 15: 181–90.
29. Watanabe Y, Konishi M, Shimada M, Ohara H, Iwamoto S. Estimation of age from the femur of Japanese cadavers. *Forensic Sci Int* 1998; 98: 55–65.
30. Dudar JC, Pfeiffer S, Saunders SR. Evaluation of morphological and histological adult skeletal age-at-death estimation techniques using ribs. *J Forensic Sci* 1993; 38: 677–85.
31. Stout SD, Lueck R. Bone remodeling rates and skeletal maturation in three archaeological skeletal populations. *Am J Phys Anthropol* 1995; 98: 161–71.
32. Cannet C, Baraybar JP, Kolopp M, Meyer P, Ludes B. Histomorphometric estimation of age in paraffin-embedded ribs: a feasibility study. *Int J Legal Med* 2011; 125: 493–502.
33. Kim YS, Kim DI, Park DK, Lee JH, Chung NE, Lee WT et al. Assessment of histomorphological features of the sternal end of the fourth rib for age estimation in Koreans. *J Forensic Sci* 2007; 52: 1237–42.
34. Cho H, Stout SD, Bishop TA. Cortical bone remodeling rates in a sample of African American and European American descent groups from the American Midwest: comparisons of age and sex in ribs. *Am J Phys Anthropol* 2006; 130: 214–26.
35. Stout SD, Dietze WH, İscan MY, Loth SR. Estimation of age at death using cortical histomorphometry of the sternal end of the fourth rib. *J Forensic Sci* 1994; 39: 778–84.
36. İscan MY, Loth SR, Wright RK. Age estimation from the rib by phase analysis: White females. *J Forensic Sci* 1985; 30(3):853–63.
37. Kranioti EF, İscan MY, Michalodimitrakis M. Craniometric analysis of the modern Cretan population. *Forensic Sci Int* 2008; 180: 110.e1–5.
38. Paine RR. Do it yourself: How to equip a basic histological lab for the anthropological assessment of human bone and teeth. *J Anthropol Sci.* 2007; 85: 213–9.
39. Harris EF, Smith RN. Accounting for measurement error: A critical but often overlooked process. *Arch Oral Biol* 2009; 54: S1, 107–17.

40. Lewis SJ. Quantifying measurement error. Current and recent research in osteoarchaology 2: Proceedings of the 4th, 5th and 6th meetings of the Osteoarchaeological Research Group. Oxford: Oxford Books, 1999: 54-5.
41. Ritz-Timme S, Cattaneo C, Collins MJ, Waite ER, Schütz HW, Kaatsch HJ, Borrman HI. Age estimation: the state of the art in relation to the specific demands of forensic practise. *Int J Legal Med* 2000; 113: 129–36.
42. Aiello L, Molleson T. Are microscopic ageing techniques more accurate than macroscopic ageing techniques? *J Archaeol Sci* 1993; 20(6): 689-704.
43. Pratte DG, Pfeiffer S. Histological Age Estimation of a Cadaveral Sample of Diverse Origins. *Can Soc Forensic Sci J* 1999; 32:155–67.
44. Weinstein RS, Bell NH. Diminished rates of bone formation in normal black adults. *N Engl J Med* 1988; 319: 1698–701.
45. Thompson DD, Gunness-Hey M. Bone mineral-osteon analysis of Yupik-Inupiaq skeletons. *Am J Phys Anthropol* 1981; 55: 1–7.
46. Ubelaker DH. Problems with the microscopic determination of age at death. Paper presented at the 1977 Meeting of the American Academy of Forensic Sciences, 1977. San Diego, CA.
47. Ubelaker DH. Estimation of age at death from histology of human bone. In: Zimmerman MR, Angel JL, editors. *Dating and Age Determination of Biological Materials*. London: Croom Helm; 1986, p. 240–7.
48. Fangwu Z. Preliminary study on determination of bone age by microscopic method. *Acta Anthropol Sinica* 1983; II: 142–51.
49. Lee U-Y, Jung G-O, Choi, SG, Kim Y-S. Anthropological Age Estimation with Bone Histomorphometry from the Human Clavicle. *Anthropologist* 2014; 17(3): 929-36.
50. Oursler MJ, Kassem M, Turner R, Riggs BL, Spelsberg TC. Regulation of bone cell function by gonadal steroids. In: Marcus R., Feldman D., Kelsey J, editors. *Osteoporosis*, New York: Academic Press; 1996, p. 237–260.
49. Tersigni M. Serial Long Bone Histology: Inter- and Intra-Bone Age Estimation. Dr. Diss. 2005. University of Tennessee, Knoxville.
51. Burr DB, Ruff CB, Thompson DD. Patterns of skeletal histologic change through time: comparison of an archaic Native American population with modern populations. *Anat Rec* 1990; 226: 307–13.
52. Pfeiffer S. Variability in osteon size in recent human populations. *Am J Phys Anthropol* 1998; 106: 219–27.

53. Bousson V, Meunier A, Bergot C, Vicaud E, Rocha MA, Morais MH et al. Distribution of intracortical porosity in human midfemoral cortex by age and gender. *J Bone Mine Res* 2001; 16: 1308–17.
54. Nelson DA, Feingold M, Bolin F, Parfitt AM. Principal components analysis of regional bone density in black and white women: relationship to body size and composition. *Am J Phys Anthropol* 1991; 86: 507–14.
55. Tersigni M. Serial Long Bone Histology: Inter- and Intra-Bone Age Estimation. Dr. Diss. 2005. University of Tennessee, Knoxville.
56. Iwaniec UT, Crenshaw TD, Schoeninger MJ, Stout SD, Ericksen MF. Methods for improving the efficiency of estimating total osteon density in the human anterior mid-diaphyseal femur. *Am J Phys Anthropol* 1998; 107: 13–24.
57. Chan AHW, Crowder CM, Rogers TL. Variation in cortical bone histology within the human femur and its impact on estimating age at death. *Am J Phys Anthropol* 2007; 132: 80–8.
58. Walker RA, Lovejoy CO, Meindl RS. Histomorphological and geometric properties of human femoral cortex in individuals over 50: Implications for histomorphological determination of age-at-death. *Am J Hum Biol* 1994; 6: 659–67.
59. Keough N, L'Abbe' EN, Steyn M. The evaluation of age-related histomorphometric variables in a cadaver sample of lower socioeconomic status: implications for estimating age at death. *Forensic Sci Int* 2009; 191: 114.e1–e6.
60. Christensen AM, Crowder CM. Evidentiary Standards for Forensic Anthropology. *J Forensic Sci* 2009; 54: 1211-1216.